

THE ROLE OF ACTGCT MOTIF IN THE EXPRESSION
OF THE MINIMAL CONSTITUTIVE ENHANCER OF THE
HUMAN PAPILLOMAVIRUS TYPE 18 IN C33A
CERVICAL CARCINOMA CELLS

CENTRE FOR NEWFOUNDLAND STUDIES

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RAGHURAM HASNADKA



The Role of ACTGCT Motif in the Expression of the
Minimal Constitutive Enhancer of the Human Papillomavirus
Type 18 in C33A Cervical Carcinoma Cells

by

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ABSTRACT

The activity of the human papillomavirus type 18 minimal constitutive enhancer (nucleotides 7508-7738) was studied in C33A cervical carcinoma cells. Unidirectional deletion mutations were performed to delineate the minimal constitutive enhancer (MCE) region that is important for the human papillomavirus type 18 expression. Maximum reduction in the activity was seen when region V (nucleotides 7673-7738) was deleted. The deletion mutant enhancer pBK8315 (nucleotides 7508-7664) expressed only 6.5 percent of the full length MCE activity. Though the expression was the least with this mutant, all the regions appeared to be a requirement for the full activity of the MCE. Gel retardation assays with the full length MCE using C33A nuclear extract showed two complexes. Gel retardation assays were performed with deletion mutants having regions I-IV (nucleotides 7508-7664) and region V using C33A nuclear extract. The slower migrating complex associated with regions I-IV, while the faster migrating complex was bound to region V. To identify the important motif of region V and thereby the factor responsible for the expression, site directed mutagenesis was performed. The ACTGCT motif at nucleotides 7679-7684 was mutated. The *in vivo* activity of

this site-directed mutant plasmid pBB155 was reduced to the extent similar to pBK8315, signifying the importance of this motif in the context of the full length MCE. When gel retardation assay was performed with the site-directed mutant using C33A nuclear extract, the faster migrating complex was abolished, while the slower migrating complex was present. The mutant MCE competed with the slower migrating complex, while the faster migrating complex was still present. The *in vitro* binding assays correlated with the *in vivo* activity in C33A cells. To further investigate the binding property of the mutant motif, UV crosslinking was performed using C33A nuclear extracts with MCE sequences from nucleotides 7667-7693. A band present with the wild type sequence was absent when the mutant sequence was used in the reaction. These experiments demonstrate the importance of the ACTGCT motif at nucleotides 7679-7684 and suggest the involvement of an ACTGCT binding protein (ABP) in the regulation of HPV 18 MCE activity.

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LIST OF ABBREVIATIONS

ABP	"ACTGCT binding protein"
AP1	activator protein 1
bp	base pairs
BPV	bovine papillomavirus
BRL	Bethesda research laboratories
BSA	bovine serum albumin
C	celsius
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
CTD	carboxyl terminal domain
DME	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
FCS	fetal calf serum
HPV	human papillomavirus
kDa	kiloDaltons
MCE	minimal constitutive enhancer

mRNA	messenger RNA
NCR	noncoding region
NF1	nuclear factor 1
ORF	open reading frame
PBS	phosphate buffered saline
RNAPII	RNA polymerase II
SV 40	simian virus 40
TEF-1	transcription enhancer factor-1
URR	upstream regulatory region
UV	ultraviolet

CHAPTER 1

Introduction

1.1 Transcriptional Regulation in Eukaryotes

The pioneering work by Jacob and Monod (1961), on the regulation of genes involved in lactose metabolism in *E. coli*, laid the ground work for the since vastly progressed research on transcriptional regulation. They demonstrated the switching on of the synthesis of the enzyme β -galactosidase as a response to the presence of the substrate, lactose. This synthesis was mediated by the increased transcription of this gene.

In contrast to prokaryotic cells, which have a single RNA polymerase enzyme, eukaryotes contain three distinct RNA polymerases. RNA polymerase I (RNAP I) transcribes ribosomal RNA, RNAP II is the polymerase for protein coding genes, whereas RNAP III transcribes 5S rRNA and tRNA genes (reviewed in Zawel & Reinberg, 1993).

The accurate initiation of transcription by RNAP II requires promoter-containing sequences upstream of the coding region (Will et al., 1979). Promoters were identified by mutational analysis of ovalbumin and adenovirus major late

gene coding sequences (Gannon et al., 1979; Corden et al., 1980). The role of sequences called enhancers, in transcription was first demonstrated by Grosschedl and Birnstiel (1980).

1.1.1 Promoter Elements in the Regulation of Transcription

The promoter sequence directs the accurate and efficient initiation of transcription by RNAP II. Promoters can be classified as proximal elements and the TATA box. In many protein coding genes, accurate transcription requires the presence of a TATA box, which is present about 25 bp upstream of the transcription start site. This directs the stable formation of the pre-initiation complex. The TATA element is believed to be responsible for determining the direction of transcription along the DNA template. Transcription is generally unidirectional. Some promoters have a CCAAT box present about 80 bp upstream of the TATA box. This was originally identified in the promoter of vertebrate globin genes (reviewed in Nussinov, 1990).

Promoters of many housekeeping genes lack TATA boxes, and instead have GC rich elements that are often within methylation-free islands (Bird, 1986). When a TATA box is not present, RNAP II is fixed at the start site for the initiation of transcription by an "initiator" element. It is a 17 base

pair sequence containing the transcription start site. This was first identified in the lymphocyte-specific terminal deoxynucleotidyl transferase gene (Smale & Baltimore, 1989).

In addition to the TATA box, a promoter also has upstream promoter elements (UPEs) which are 8-12 bp long sequences (reviewed in Maniatis et al., 1987). The UPEs function to increase the rate of transcription, while the TATA box ensures accurate initiation of transcription. GC box and CCAAT box are present in many genes transcribed by RNAP II. The strength of the promoter is believed to depend on the number of UPEs.

1.1.2 The Role of Enhancer Elements

Enhancers regulate promoters from a distance, in an orientation independent fashion. The enhancer element was first identified in SV40 (Banerji et al., 1981). This 200 bp DNA segment was positioned 200 bp upstream of the start site and could enhance transcription by 100 fold. Enhancers can activate transcription when present in either orientation. The basic unit of an enhancer element is called enhanson, which is an individual binding site for transcriptional activator proteins. By interchanging or duplicating the units, new enhancer elements can be created (Ondek et al., 1988; reviewed in Muller et al., 1988).

Enhancers are believed to increase the rate of transcription by increasing the density of RNA polymerase enzyme over a linked gene (Weber & Schaffner, 1985). The possible mechanisms to achieve activated transcription could be by tracking or looping of DNA (reviewed in Wang & Giaever, 1988). According to the scanning model proposed by Moreau et al. (1981), the RNAP II, after binding to the enhancer, slides in both directions along the DNA until it makes contact with the start site. The looping model proposes the interaction of enhancer and promoter through bound proteins, leading to the looping out of the intervening DNA sequences. Tracking can also induce DNA looping. The DNA structure is important for the action of enhancers. Deletion of DNA corresponding to a half helical turn is known to disrupt the enhancer activity, whereas removal of multiples of ten bases between SV40 enhancer and its promoter had no effect on enhancer activity (Schleif, R., 1988).

Enhancers can be categorized into two classes. The enhancers that respond to changes in the environment are called inducible enhancers. The enhancers active in specific tissues are called tissue-specific and the ones expressed at certain stages of development are temporal enhancers. Inducible enhancers have been known to be present in genes including heat shock, metallothionein genes. The activation of steroid responsive genes by the binding of steroid receptor

proteins is well understood (reviewed in Maniatis et al., 1987; Pelham, 1982).

The expression of immunoglobulin (Ig) genes in lymphoid cells is a well studied example for tissue specific expression. Immunoglobulins are expressed in B cells and lymphoid cells. The synergistic interaction between the immunoglobulin promoter and enhancer controls the tissue-specific expression of Ig genes (Muller et al., 1988). The high level expression of insulin genes in pancreatic endocrine cells is also controlled by the enhancer element. Tissue-specific enhancers are involved in the expression of elastase and chymotrypsin genes in the exocrine cells of pancreas (Edlund et al., 1985; Boulet et al., 1986).

1.1.3 The Initiation Complex

Accurate transcription by RNA polymerase II requires a preinitiation complex of a multifactor, nucleoprotein assembly. This involves the assistance of auxiliary factors, referred to as general transcription factors (GTFs). At least five general transcription factors (TFIIA, TFIIB, TFIID, TFIIE and TFIIF) are required to form an active initiation complex (Dynlacht et al., 1991; Buratowski et al., 1989).

Transcription factor IID (TFIID) was first identified in *Drosophila* nuclear extracts (Parker & Topol,

1984) . It is the only GTF having DNA binding activity. TFIID binds to the TATA box of RNAP II promoters and directs the orderly assembly of RNAP II and other GTFs to form the initiation complex (reviewed in Conaway & Conaway, 1993). This complex is capable of initiating a basal level of transcription. To mediate transcriptional regulation by upstream factors, TATA box binding protein (TBP) associated factors are required (reviewed in Tjian & Maniatis, 1994). In eukaryotes, only one TFIID molecule exists with the exception of *Arabidopsis*, where two genes encoding TFIID were found. TFIID appears to recognize variant TATA motifs with different affinities to mediate different responses. The association of other factors might enable this versatility (Hernandez, 1993; Gasch et al., 1990). The inner surface of TFIID interacts with the minor groove of the TATA box, leading to distortions in the DNA. Other factors bind to the outer surface of TFIID (Klug, 1993).

TFIIA is known to bind to TFIID-promoter complex, early in the initiation process. TFIIA is believed to counteract the negative regulators by competition, and stabilize the TFIID promoter complex (reviewed in Conaway & Conaway, 1993). TFIIA functions by recruiting RNAP II into the initiation complex and also binds to the TFIID-DNA complex directly. It is also believed to mediate interactions between the initiation complex and upstream activators. TFIIA binding

to the initiation complex is speculated to be the rate-limiting step in the initiation (Buratowski, 1989; Lin & Green, 1991). TFIIE is required for accurate *in vitro* transcription by RNAP II. TFIIE binds to the initiation complex after RNAP II-TFIIF are bound to TFIIDAB (reviewed in Zawel & Reinberg, 1993). TFIIF functions in bringing the RNAP II enzyme to the initiation complex. It is also involved in the elongation of transcription (Buratowski et al., 1989; Bengal et al., 1991).

A recent study suggests that RNAP II may form a multicomponent complex with some initiation factors before binding to the promoter site (Koleske & Young, 1994). It is believed that components of the initiation complex such as TFIIA, TFIIB, TFIIE and TFIIH act as potential regulatory targets for activators. The activation domain of VP16 is known to interact with TFIIB (Tjian & Maniatis, 1994).

RNA polymerase II initiates transcription of eukaryotic protein coding genes with the assembly of the pre-initiation complex. The RNAP II of HeLa cells contains 10 subunits with molecular weights ranging from 240 to 10 kDa. A unique C-terminal domain (CTD) is present in the largest subunit of eukaryotic RNAP II, which is absent in prokaryotic RNAP or eukaryotic RNAP I and RNAP III. This CTD has multiple repeats of consensus heptapeptide sequence containing serine and threonine residues, which can be phosphorylated (reviewed

in Zawel & Reinberg, 1993). The CTD is known to bind at the promoter site by intercalating into DNA (Suzuki, 1990). The CTD is believed to function by mediating transcriptional activation through protein-protein functions as seen in activation by Gal 4 (Allison & Ingles, 1989). The RNAP II is in its unphosphorylated form when bound to the DNA initiation complex, at the time of transcription initiation. The CTD has to be phosphorylated to be capable of transcript elongation. TFIIF is believed to contain a CTD kinase activity responsible for the phosphorylation (Laybourn & Dahmus, 1990; Arias et al., 1991).

1.1.4 Transcription Factors in the Regulation of Transcription

The regulation of gene expression is controlled by the binding of nuclear factors to the conserved sequences present in the regulatory region of the gene. These transcription factors bind to regulatory elements present within several hundred base pairs from the initiation site. This regulation can be mediated by the different transcription factors binding to their specific conserved sequences. Broadly, the transcription factors are classified into ubiquitous factors and tissue specific factors (Polyanovsky & Stepchenko, 1990). Deletion analysis of cloned transcription

factors have identified regions of the factors responsible for DNA binding and transcriptional activation (reviewed in Mitchell & Tjian, 1989).

The DNA binding zinc finger motif has about 30 amino acids with two cysteine and two histidine residues that stabilize the binding by a tetrahedral coordination with Zn^{2+} ion. This motif is found in transcription factor Sp1, which activates transcription by binding to GC box present in the promoter. Sp1 has two glutamine rich regions which are responsible for the transcriptional activation by Sp1 (Courey & Tjian, 1988). The zinc finger motif is also seen in the DNA binding domain of steroid hormone receptors. It was first identified in RNAP III transcription factor TFIIIA which regulates transcription of 5S RNA gene (reviewed in Mitchell & Tjian, 1989).

The homeodomain (HD) present in the ubiquitous Oct-1 factor recognizes the octomeric motif ATTTGCAT. This domain stretches over 60 amino acids. The HD is present in homeotic genes of *Drosophila* and vertebrates (Standt et al., 1988; reviewed in Mitchell & Tjian, 1989).

The transcription-replication factor CTF/NF-1 binds the GCCAAT box present in many genes. The DNA binding domain is localized in its NH_2 -terminal end which has a basic region ideal for α -helical structure. The proline rich region present in its COOH-terminus, activates transcription in

association with zinc fingers of Sp1 (reviewed in Mitchell & Tjian, 1989).

The transcription factor C/EBP has a region of 35 amino acids in which every seventh amino acid is a leucine. Similar regions are present in transcription factors GCN4, Myc, Fos and Jun. These leucine rich regions form an α -helix, with the leucines occurring every two turns on the same side, resembling a leucine zipper. This region facilitates dimerization of two protein molecules such that the adjacent basic rich region can bind to DNA (Landschultz et al., 1988).

The activation domain of transcription factors is distinct from their DNA binding domain. Though DNA binding domain is essential for transcriptional activation, it is not sufficient without the activation domain of the factor. The yeast transcription factor Gal4 has two acidic regions which can form amphipathic α -helical structure, with negative charges on one side of the helix available for contact with another protein (reviewed in Mitchell & Tjian, 1989; Hope & Struhl, 1986).

1.2 Papillomaviruses

Papillomaviruses are small, non enveloped, icosahedral double stranded circular viruses containing DNA of

about 8,000 base pairs. They belong to the sub-family papillovirinae (Fields, 1990).

The study of papillomaviruses gained importance after the demonstration of the role of cottontail rabbit papillomavirus in the etiology of epithelial neoplasia. Later, the association of human papillomavirus with epidermodysplasia verruciformis established the causative role of papillomaviruses in humans (reviewed in Sousa et al., 1990).

More than 65 types of HPVs have been classified based on the species of origin and the lack of homology with other papillomaviruses from the same species (Payne et al., 1993; DeVilliers, 1989). Papillomaviruses are highly species-specific and tissue-specific. Vegetative growth of the viruses is seen only in the nucleus of differentiated keratinocytes of epithelia. The viral particles are found only in the superficial layers of the epithelium (Broker & Botchan, 1986; Pfister, H., 1987).

Human papillomaviruses can be categorized as low risk and high risk HPVs. HPV 6 and HPV 11 are present in benign warts, exophytic condylomas but absent in high grade neoplasias. The high risk HPV 16 and HPV 18 are predominantly seen in high grade neoplasias of the anogenital region and invasive cancers of the cervix, vulva, penis and anus (Sang & Barbosa, 1992). Transfection of DNA from HPV 16 and HPV 18

into primary human keratinocytes can immortalize the cells, whereas HPV 6 and HPV 11 DNA fail to immortalize cells, leading to senescence (Woodworth et al., 1989).

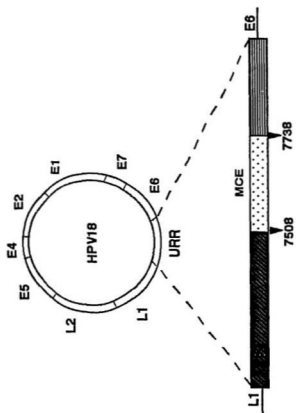
1.2.1 The HPV Genome

The Bovine Papillomavirus 1 is taken as the prototype virus for PVs. The genome has ten open reading frames (ORFs), 8 early ORFs and 2 late ORFs as in fig 1. The ORFs are located on one strand of the viral DNA and transcripts are encoded from this strand. The L ORFs express only in terminally differentiated cells, whereas the E ORFs are expressed in non-productively infected and transformed cells (Chen et al., 1982; Baker & Howley, 1987). The HPV DNA is found integrated into cellular DNA in most HPV positive cervical carcinoma cell lines, while HPV DNA is present extrachromosomally in benign lesions (Durst et al., 1985).

The E1 ORF is the largest ORF and is well conserved in all the papillomaviruses. The E1 ORF encodes a modulator protein which is essential for the stable establishment of plasmid DNA. The regulator E1-R, encoded by E1 ORF, is involved in the replication of DNA. The E1-R gene is believed to be involved in DNA amplification as seen in the growth arrested cells (Lusky & Botchan, 1986; Burnett et al., 1989; Ustav & Steinland, 1991).

Fig.1.

Schematic diagram of HPV 18 genome organization. The upstream regulatory region (URR) is situated between Late (L) 1 open reading frame (ORF) and Early (E) 6 ORF. The 230 bp minimal constitutive enhancer (MCE) is present in the URR (Butz & Hoppe-Seyler, 1993).



The disruption of HPV 16 E1 gene is believed to increase the viral immortalization capacity, since a HPV 16 DNA clone isolated from a cervical carcinoma contained a deletion, resulting in a frameshift mutation of the E1 gene. Mutation of either the E1 or the E2 gene is believed to impart a high immortalization efficiency for HPV 16 (Romanczuk & Howley, 1992).

The E2 gene products are involved in regulating viral transcription and replication. The BPV-1 E2 transactivator stabilizes the replication pre-initiation complex by stimulating the binding of E1 replication protein to the replication site and thereby activates DNA replication (Yang et al., 1991). The E2 ORF of BPV 1 encodes a transactivator from full length ORF and two transcriptional repressors from the 3' end of ORF. The c-terminal region of the transactivator and repressor proteins is capable of forming dimers to bind to DNA. This dimerization between transactivator and repressor may lead to the formation of inactive heterodimers (McBride et al., 1989). The BPV-1 E2 transactivating protein can stimulate HPV 18 long control region and also repress the same. The binding of E2 protein between the CAAT and TATA element of the HPV 18 long control region may lead to sterical hindrance of the initiation complex (Thierry & Yaniv, 1987). E2 protein is known to compete with Sp1 and repress the E6 promoter of HPV 16 (Tan et

al., 1991). The role of E2 ORF in the immortalization of cervical keratinocytes has been suggested, since HPV 16 with a mutation in the E2 ORF was unable to immortalize (Storey et al., 1992).

HPV 16 E4 protein has been reported to be present in cutaneous warts. This expression confined to the areas of epithelium expressing the L1 capsid protein. Unlike the classical DNA virus early proteins, the HPV E4 gene products are believed to play a role in virus maturation (Crum et al., 1990; Doorbar et al., 1986).

The E5 protein is the smallest viral transforming protein, with a molecular mass of 7 kDa containing 44 amino acids. The ability of BPV to transform fibroblasts *in vitro* is due to the E5 protein. The E5 gene from both BPV and HPV 16 can transform established epidermal keratinocytes. The transforming activity is encoded in its c-terminal domain (reviewed in Sousa et al., 1990; Leptak et al., 1991).

1.2.2 HPVs in the Etiology of Cervical Cancer

Research on the role of HPVs in the causation of female genital tract infections has been intensively pursued over the years. The association of HPVs with benign papillomatous lesions of the skin, plantar warts, flat warts, genital condylomas, epidermodysplasia verruciformis is well documented

(Howley, 1982). The exposure of uterine cervix to HPV, leads to the formation of genital wart like lesions. The precancerous lesions of genital warts are referred to as cervical intraepithelial neoplasia (CIN). The lesions appear predominantly at the junction of the squamous and columnar epithelium, known as the transformation zone. Here, the columnar epithelium is replaced by metaplastic squamous epithelium. The sexual transmissibility of these warts and the high incidence of this condition led to extensive studies on the pathogenesis of CIN (Nuova et al., 1987).

The oncogenic types of HPVs are known to be causative agents in the pathogenesis of cervical cancer. More than 90% of the cervical carcinoma biopsies reveal the presence of HPV DNA. The frequent detection of mRNA transcripts for early proteins E6 and E7 of HPV 16 and 18, strongly suggests the role of these proteins in the transformation of cells. These early proteins have been detected from cervical carcinoma cells *in vitro*. The inability to demonstrate these proteins *in vivo* is believed to be due to the low abundance of the proteins or masking of E7 epitopes in the nucleus by binding with cellular proteins, oligomerization or self folding (zur Hausen, 1989; Smotkin & Wettstein, 1986; Grissmann, 1992; Kanda et al., 1991).

Supportive evidence for the oncogenic potential of the high risk HPVs has accumulated in abundance. Transfection

of HPV 16 and 18 DNA into primary human keratinocytes leads to immortalization of the cells, while the low risk type HPVs fail to immortalize keratinocytes. The immortalized cells show aneuploid karyotype like the cells from premalignant cells (Woodworth et al., 1989; Fu et al., 1983). When cells immortalized with high risk HPV DNA were grafted beneath a skin-muscle flap of athymic mice, the grafts exhibited dysplastic morphology and molecular alterations in gene expression, characteristic of intraepithelial neoplasia (Woodworth et al., 1990).

The HPV DNA of high risk types 16 and 18 are frequently integrated in cervical cancers and HPV positive cervical carcinoma cell lines. The viral DNA integration has been observed to occur in the general vicinity of known oncogenes. In HeLa cells the integration of HPV 18 DNA occurs at a site 50 kbp from the c-myc locus on chromosome 8. However the significance of this integration pattern is not clear. The viral DNA from low risk HPVs are usually present extrachromosomally in episomal form (Sang & Barbosa, 1992; Durst et al., 1985; Durst et al., 1987).

The genes directly involved in the transformation of primary human embryonic fibroblasts by HPV 16 were identified to be E6 and E7, by transfecting subgenomic fragments of HPV 16 DNA under the control of SV40 promoter (Watanabe et al., 1989). Similarly, it was shown that full length HPV 16 E6 and

E7 genes are required and sufficient for transformation of primary human keratinocytes (Halbert et al., 1991).

The knowledge about the association of E7 with pRB and E6 binding to p53, gave information about the possible mechanism of tumorigenicity by E6 and E7. p53 mutations have been observed in metastasized cervical carcinomas, indicating the role of p53 in the pathogenesis of cervical cancer. It is hypothesized that specific interactions of E6 and E7 with p53 and pRB abrogate the functions of these proteins in cervical cancer by mutation of genes or by the binding itself. The E7 proteins encoded by the high risk HPVs bind pRB with higher affinity than the E7 proteins of the low risk HPVs, suggestive of the reason for the differential pathogenicity between the high risk and low risk HPV types (Dyson et al., 1989; Crook & Vousden, 1992; Schaffner et al., 1991; Munger et al., 1991). The role of glucocorticoid hormones in the transformation process has been studied. The inhibition of the hormone dependent transformation of HPV 16 and ras was seen when baby rat kidney cells were treated with the hormone antagonist RU 486 (Pater et al., 1988; Pater & Pater, 1991).

1.2.3 The Regulatory Regions of HPV Genome

In most cervical carcinomas and tumor cell lines, HPV DNA is found integrated into the host genome leading to

disruption of the E1 and E2 ORFs. The absence of these gene products is believed to cause deregulation of E6 and E7 expression, since E2 is the known repressor of E6 and E7 transcription. Thus, the expression of integrated HPV DNA is not regulated by HPV encoded proteins. After integration into host genome, E6 and E7 are regulated by host factors. Unlike the integrated DNA, episomal DNA is regulated by HPV encoded proteins (reviewed in McBride et al., 1991).

In the absence of any viral gene product, HPV 18 noncoding region (NCR) can function as a transcription control element in many cervical cell lines (Thiery & Yaniv, 1987). In all papillomaviruses, the NCR/upstream regulatory region (URR) has the replication and transcription regulatory regions. This is believed to regulate expression of the HPV ORFs. This NCR/URR is present between the end of the late ORF and the start of the E6 gene (Garcia-Carranca et al., 1988). There has been much interest in investigating the role of the NCR due to its ability to direct transcription of the downstream ORFs.

1.2.4 Role of the MCE in the Expression of HPV 18

The URR of HPV 18 has three distinct enhancer elements. Two of the enhancers are inducible by HPV 18 gene products, while the third enhancer does not require any HPV 18

gene product. This enhancer is termed as the minimal constitutive enhancer (MCE). The MCE is 230 bp long, present between the two inducible enhancers (see Fig 1.). The 388 bp enhancer present adjacent to the L1 ORF is the least conserved enhancer. This enhancer is believed to be involved in the viral gene expression when HPV 18 is in the episomal form. When integrated into host genome, as in malignant transformation of the cells, the HPV genome is not under the control of the E2 gene product. Viruses expressing only the E6 and E7 genes are known to be better transforming agents relative to the entire viral genome. The 237 bp enhancer element is present close to the E6 ORF. This element has TATA and CAAT boxes for RNAP II binding (Gius et al., 1988; Swift et al., 1987; Ward et al., 1989).

The constitutive enhancers of HPV 11, 16 and 18 are capable of activating expression of heterologous promoters to high levels in squamous epithelial cells in transient transfection assays. This suggests the role of the constitutive enhancers in the tissue tropic expression of HPVs (Mack & Laimins, 1991). Epidemiological studies indicate that HPV 18 is a particularly high risk virus. The incidence of tumor recurrence is higher with HPV 18 than with HPV 16. The virus is found to be associated more commonly with invasive cancers. The onset age is usually less by 10 years with HPV 18, compared to cancers caused by HPV 16. Lesions with

adenocarcinoma and small cell carcinomas are more associated with HPV 18. Infection with HPV 18 is therefore believed to cause rapidly progressive cancers of the cervix (Stoler, M.H. et al., 1992). When chimeric constructs of HPV 16, 18, E6 and E7 ORFs with SV40, HPV 16 and HPV 18 URRs were used in transformation assays, the construct with HPV 18 URR showed the highest immortalization efficiency. This indicates that the URR upstream of E6 and E7 is the major determinant of the differential immortalization activities of HPV 16 and HPV 18 and HPV 18 URR/E6/E7 is more efficient than the analogous region of HPV 16.

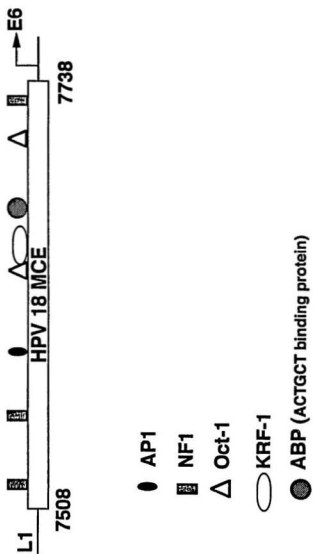
1.2.5 Cellular Factors Bind to the HPV 18 MCE

DNA-protein binding assays have demonstrated the binding of cellular factors to the HPV 18 MCE, as shown in fig 2. The importance of the MCE in the regulation of HPV expression in transformed cells has attracted extensive research in understanding this mechanism. Though the vast body of data has aided in understanding this intricate process, an unambiguous explanation is not available.

Three nuclear factor 1 (NF1) motifs have been identified in the HPV 18 MCE. NF1 is a DNA binding protein recognizing the CCAAT box. It is known to be important in the activation of cellular genes and DNA replication.

Fig 2.

Diagrammatic representation of cellular factors binding to the 230 bp HPV 18 minimal constitutive enhancer (nucleotides 7508-7738). AP1, NF1, Oct- 1 and KRF-1 are the factors known to bind to the MCE (Hoppe-Seyler and Butz,1994). The proposed binding of the "ACTGCT binding protein" is also depicted.



Experiments with the HPV 16 URR indicated the importance of NF1 sites in the regulation, in association with AP1. The glucocorticoid responsiveness of HPV 16 enhancer is said to be partially dependent on the presence of NF1. Though ubiquitous in distribution, NF1 is argued to be important in the cell-specific function of the HPV 16 enhancer. However, the contribution of NF1 in the control of expression of HPV 18 URR is found to be only marginal (Chong et al., 1990; Apt et al., 1993; Butz & Hoppe-Seyler, 1993).

Both HPV 16 and HPV 18 URRs have activator protein 1 (AP1) elements. While HPV 16 has 3 binding sites, HPV 18 MCE has one element and another in an inducible enhancer. AP1 is a heterodimer of protooncogene products of *jun* and *fos*. AP1 is a transcription factor isolated from HeLa cells and is identical to the human *jun* gene product. AP1 is believed to be important in the expression of HPV 18 URR. In terminally differentiated cells, *jun B* homodimers are the AP1 elements involved. A recent study suggests the possible role of an unidentified factor present in C33A cells that is essential for activation of HPV 18 expression in association with *jun B* (Chong et al., 1991; Vogt & Bos, 1990; Thierry et al., 1992).

The octomer-binding transcription factor-1 (oct-1) has two low affinity binding sites on HPV 18 URR. One oct-1 site overlaps with the recognition sequence of KRF-1 and

represses enhancer activity. Mutation of the oct-1 site at the 3'-end of HPV 18 MCE did not show an effect on the activity. Oct-1 is abundantly expressed in proliferating transforming cells. The site present in HPV 16 URR exhibits opposite effects in cervical and non-cervical cells. While it inhibits gene expression in non-cervical cells, a novel octomer binding factor present in cervical cells is believed to activate expression by displacing oct-1 (Hoppe-Seyler & Butz, 1994; Morris et al., 1993).

KRF-1 is a keratinocyte-specific transcription factor, known to bind the HPV 18 MCE. There is no known KRF-1 binding site on the HPV 16 URR. The specific distribution of this factor in keratinocytes is argued to be the reason for HPV 18 expression in keratinocytes. However, HPV 16 which is also a tissue tropic virus, does not have any control in its expression, from KRF-1 (Mack & Laimins, 1991; Hoppe-Seyler & Butz, 1994).

The HPV 16 URR has three motifs for transcriptional enhancer factor (TEF)-1, which is present in keratinocytes and cervical carcinoma cells (Ishiji et al., 1992). A cellular zinc-finger protein YY1 is believed to have a repressing role on HPV 18 expression. The deletion or mutation of this site in cervical carcinomas is hypothesized to be the mechanism in the development of carcinoma (Hoppe-Seyler & Butz, 1994).

1.3 Objectives of the Study

With the knowledge about the important role of the HPV 18 MCE in cervical carcinoma caused by the high risk HPV 18, the aim of this study was to understand the molecular mechanism involved in the regulation of HPV 18 MCE using C33A cervical carcinoma cells. The studies were performed using C33A cells, since they do not contain HPV DNA, thereby ruling out the involvement of virally encoded factors. This provides an environment to investigate the activity of HPV 18 MCE and its regulation by the host cellular factors.

The working hypothesis was that the identification of the MCE region most significant in regulating the activity of HPV 18 would be the first step towards meeting the objective. This region would then give information about the protein(s) binding to this fragment and the role in the regulation of HPV18 expression. The successful delineation of the MCE would then direct further attempts at identifying the protein(s) most important in the tissue tropism of the virus.

Earlier work by Dr. Pater's lab had identified five DNaseI protected regions in the MCE of HPV 18, labelled as regions I to V (Nakshatri et al., 1990). The possible role of an ACTGCT sequence present at nt 7679 to 7684 of region V of the HPV 18 MCE was speculated by the authors. The absence of

this sequence in the non-oncogenic HPV 11, while also present at nt 7545 to 7550 of region I in HPV 18 MCE and at nt 7579 to 7584 of HPV 16 MCE was an interesting observation. No known transcription factor is known to recognize this sequence (Faisst & Meyer, 1992).

Generation of unidirectional deletions of the MCE from either end using exonuclease III was the first step in this direction. The deleted regions of the HPV 18 MCE would then be cloned upstream to the reporter chloramphenicol acetyl transferase (CAT) gene. The *in vivo* activities of these deletion mutants would be compared with the full length HPV 18 MCE by performing CAT assays using C33A cell extracts.

The aim of the study was also to identify factor(s) binding to the important region by DNA-protein binding assays. With the information from the deletion mutants the next step would be to perform site directed mutagenesis and identify the motif of HPV 18 MCE important and thereby the factor that is involved in the regulation.

CHAPTER 2

Materials and Methods

2.1 Materials

Dulbecco's modified Eagle's (DME) medium was prepared according to Current Protocols in Molecular Biology by Wiley Interscience. Fetal calf serum (FCS), trypsin-EDTA solution were purchased from Gibco-BRL. The C33A cell line was maintained in the laboratory.

Restriction endonucleases were ordered from Gibco-BRL with their respective 10x React buffers. T4 DNA ligase and 5x premix, exonuclease III, mung bean nuclease, calf intestine alkaline phosphatase were also purchased from Gibco-BRL. Nick translation kit was obtained from Gibco-BRL. Reverse transcriptase was supplied by Life Science.

α [³²P]dCTP and [³⁵S]dATP were purchased from Amersham. Nick columns were obtained from Pharmacia. Kodak was the supplier of thin layer chromatography (TLC) plates and x-ray films. Acetyl coenzyme A was obtained from Sigma and so was poly(dI-dC).poly(dI-dC) double strand, sodium salt from Pharmacia.

Muta-Gene Phagemid *in vitro* Mutagenesis kit was purchased from Bio Rad. Sequenase version 2.0 T7 DNA Polymerase Sequencing kit was obtained from USB. Synthetic oligonucleotides were purchased from General Synthesis and Diagnostics, Toronto and University Core DNA Services, University of Calgary, Calgary. 5-Bromodeoxyuridine was supplied by Sigma.

Recombinant plasmid pT81 was constructed by Marshall et al. (1989). The HPV 18 minimal constitutive enhancer was cloned to pUC19 by H. Nakshatri. Plasmids pA10cat and pSV2 cat were provided by B. Howard.

2.2 Cell Culture of C33A Cells

C33A cells were maintained in DME medium containing 10% heat inactivated FCS. For culturing purposes, a frozen vial of C33A cells was thawed in a 37°C water bath, centrifuged at 1,000 RPM with DME and 10% FCS and the supernatant was discarded. The pellet of cells was then resuspended with the medium and plated in a tissue culture plate. After overnight incubation at 37°C the medium was discarded and the cells were washed with PBS and trypsinized. After adding DME the cells were plated at 1:3 in tissue culture plates. The cells were maintained in culture by passaging on alternate days, when the plates were confluent.

2.3 Recombinant Plasmids

The plasmid pA10cat was used in transfections as a negative control in chloramphenicol acetyl transferase (CAT) assays. This lacks enhancer sequences but has 21 base pair repeats and TATA box of SV40 cloned upstream of the chloramphenicol acetyl transferase gene (Laimins et al. 1982).

The plasmid pSV2cat (Gorman et al. 1982) has SV40 enhancer sequences and hence used as a positive control in CAT assays.

The recombinant plasmid pT81 (fig. 3) was constructed by Marshall et al. (1989). This has the 230 base pair HPV 18 minimal constitutive enhancer (nucleotides 7508-7738) cloned at the Bgl II site upstream to the 21 base pair repeats of the plasmid pA10cat, which has the chloramphenicol acetyl transferase reporter gene.

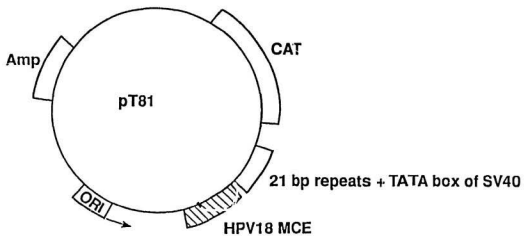
2.3.1 Construction of Deletion Mutant Plasmids

Unidirectional nested deletion of the 230 base pair long HPV 18 minimal constitutive enhancer was generated as described previously (Henikoff, 1982), with modifications as in the Strategene[®] manual. The HPV 18 MCE cloned at the Xba I site of plasmid pUC19 was used for this purpose.

Fig 3.

Schematic diagram of recombinant plasmid pT81. Amp is the ampicillin resistance gene, CAT is the chloramphenicol acetyl transferase reporter gene, HPV 18 MCE has nucleotides 7508-7738 (Marshall et al., 1989).

Schematic diagram of pT81



The double strand DNA specific enzyme exonuclease III digests single strand DNA from a 3' end only and cannot digest a blunt end or 3' overhanging end. To create unidirectional deletion from region IV end towards region I of the MCE, restriction enzymes BamH I and Kpn I were used to linearize the plasmid. The deletion mutants created with this batch were labelled as pBK, according to the different time period of the reaction. The extent of exonuclease III deletion was controlled by the duration of the enzymatic digestion and determined by sequencing (Sanger et al., 1977).

The linearized DNA was taken at the rate of 5 µg/time point in 25 µl of 1x exo III buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 10 µg/ml tRNA). The reaction was done at room temperature by adding 5 units of exo III enzyme per µg of linearized DNA. At regular time intervals, aliquots of digested DNA were added to tubes with 1x mung bean buffer (30 mM sodium acetate, pH 5.0, 50 mM NaCl, 1 mM ZnCl₂, 50% glycerol) and placed on dry ice. The tubes were then heated at 68°C for 15 minutes and placed on ice. Fifteen units of mung bean nuclease diluted in 1x mung bean dilution buffer (10 mM sodium acetate, pH 5.0, 0.1 mM zinc acetate, 1 mM cysteine, 50% glycerol) was added to each tube and incubated at 30°C for 30 minutes. The reaction was stopped by adding 10 µl of 1 M Tris-HCl, pH 9.5, 20 µl 8 M LiCl, 4 µl of 20% SDS. The mung bean protein was removed by phenol:chloroform extraction. The

linearized deletion mutants were recircularized by overnight incubation at 4°C after adding 2 units of T4 DNA ligase and ligation premix.

To create unidirectional nested deletions from region I towards region V, the pUC19 with HPV 18 MCE was linearized by digesting with Sal I and Pst I. The exonuclease III reaction was performed as explained. The mutants so generated were designated as pSP.

The deleted fragments of the HPV 18 MCE thus created were digested with EcoR I and Hind III, blunt ended and cloned into plasmid pA10cat at its Bgl II site. Plasmids pBK8315, pBK575 and pBK8110 had fragments from nucleotides 7508-7664, 7508-7640 and 7508-7589 respectively. Similarly, plasmids pSP725, pSP936 and pSP9913 had nucleotides 7596-7738, 7632-7738 and 7660-7738 respectively. The deletion fragments are depicted in fig. 4.

2.3.2 Construction of Mutant Plasmid by Site Directed Mutagenesis

The probable role of the ACTGCT sequence (nt 7679-7684) was speculated by Nakshatri et al., (1990). The sequence of the mutation to be generated was designed such that no spacing change and no known transcription factor binding site would be created as listed in the "compilation of vertebrate-

Fig 4.

The schematic representation of the deletions and site directed mutant.

pT81: nucleotides 7508-7738

pBK8315: nucleotides 7508-7664

pBK575: nucleotides 7508-7640

pSP725: nucleotides 7596-7738

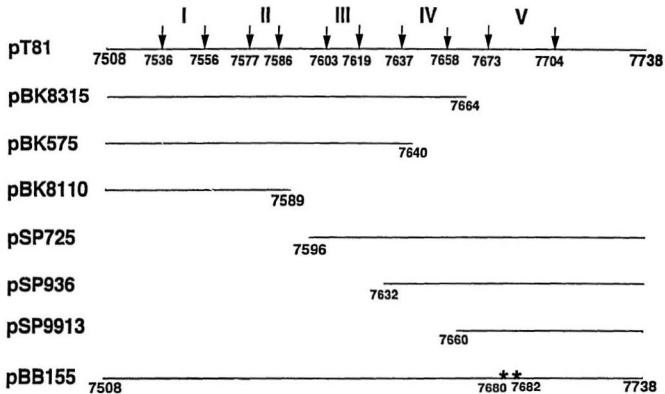
pSP936: nucleotides 7632-7738

pSP9913: nucleotides 7660-7738

Mutant pBB155 has a G at nucleotide 7680 instead of C present in pT81 and nucleotide 7682 is changed from G to A.

The arrows represent boundaries of the regions I-V.

The asterisks denote the mutated nucleotide generated by site-directed mutagenesis.



encoded transcription factors" (Faisst & Meyer, 1992). To enable an efficient and easier screening of the mutant clones, a mutant oligonucleotide was designed to create the recognition site AGTACT for the restriction enzyme ScaI in place of the sequence ACTGCT present in the wild type HPV 18 MCE.

Wild type HPV18 enhancer

nt 7667-5'-CTAAGTAATAAA**ACTGCT**TTTtaggcac-3'-7693

Mutant HPV18 enhancer

nt 7667-5'-CTAAGTAATAAA**AGTACT**TTTtaggcac-3'-7693

To generate the desired mutations of two nucleotides in region V, the Muta-gene[®] Phagemid *in vitro* Mutagenesis kit was used. The kit was purchased from Bio Rad. The plasmid pUC19 with HPV 18 MCE and vector plasmid pTZ19U were digested with Kpn I and Pst I. The HPV 18 MCE fragment and the linearized vector were eluted on S and S[®] NA45 membrane using the protocol provided therein. The eluted fragments were then ligated by incubating overnight at 16°C with T4 DNA ligase and ligase buffer. This plasmid was used to extract uracil-containing phagemid DNA by transforming into CJ 236 cells.

A synthetic oligonucleotide 5'-GTGCCTAAAAGTACTTTTATTACTTAG-3' was used as the primer to generate the desired mutation. In the mutant HPV 18 MCE, nucleotide 7680 was a G instead of the wild type C, and nucleotide 7682 was converted

to an A in place of a G (fig 4) without altering the rest of the full length of MCE. The mutation created a new recognition site for the restriction enzyme Sca I and could be screened accordingly. The mutation was determined also by dideoxy sequencing (Sanger et al., 1977).

The Sma I - Hinc II fragment of the mutated MCE was cloned into the Bgl II site of pA10cat to be designated as pBB155.

2.4 Transfection of C33A Cells

Transfections were performed by the calcium-phosphate method (Graham and Vander Eb, 1973). Eight hours before transfection, confluent plates of C33A cells were passed at 1:3 dilution. 10 µg of plasmid DNA in 50 µl of 0.1 X TE buffer was added to 62 µl of 2M CaCl₂ and made to a volume of 500 µl with distilled water. A fine precipitate of DNA was formed by mixing with 500 µl of 2X HBS (0.28 M NaCl, 0.05 M HEPES, 2.8 mM Na₂ HPO₄ pH 7.1) under gentle bubbling of nitrogen. The precipitates were let to stand for 10 minutes before adding to the plated cells. The plates were then incubated at 37°C in a 3% CO₂ incubator. After 4 hours of incubation, the medium was discarded and 1.5 ml of 15% glycerol in 2X HBS was added to the plates. The cells were washed with DME after glycerol shock for one minute. Traces

of glycerol were removed from the plates by three washes with DME. The cells were maintained at 37°C with fresh DME containing 10% calf serum for 48 hours before harvesting (Gorman et al. 1982).

2.5 Preparation of Cell Lysates

The cells were washed three times with PBS before adding 2 ml of Tris-EDTA-NaCl solution (0.04 M Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl). Cells were harvested with rubber policeman and transferred to eppendorf tubes. The cells were pelleted by centrifugation at 4°C for 30 seconds. After discarding the supernatant, the cells were stored frozen at -70°C until further use.

Cell extracts were prepared by freeze-thawing the pellet. After thawing at 37°C, the cells were resuspended in 100 µl of 0.25 M Tris-HCl, pH 7.8. The tubes were then placed on liquid nitrogen for 5 minutes. The cells were subjected to three freeze-thaw cycles with intermittent vortexing. After centrifugation at 4°C for 5 minutes in an eppendorf microfuge, the supernatants were collected in fresh tubes.

2.6 Chloramphenicol Acetyl Transferase Assay (CAT)

The CAT assay protocol described by Gorman et al. (1982) was followed to estimate the *in vivo* activity of the

plasmids. The assay mixture was made to a final volume of 100 μ l with 10 μ l of cell extract, 1 μ l 14 C chloramphenicol, 10 μ l of 4 mM acetyl coenzyme A, 45 μ l of 1 M Tris-HCl, pH 7.8 and 34 μ l of distilled water. The tubes were then incubated at 37°C for an hour. 500 μ l of ethylacetate was added to stop the reaction and extract chloramphenicol. The tubes were vortexed for 30 secs and then centrifuged at room temperature for 1 minute. The supernatant organic layer was transferred to fresh tubes and vacuum dried in a Savant aspirator.

The dried organic layer was dissolved in 15 μ l of ethylacetate and spotted on silica gel thin layer plates to be run with chloroform-methanol (95:5) in an enclosed glass jar. Acetylated and non-acetylated forms of chloramphenicol were separated by ascending chromatography. The TLC plates were exposed to Kodak x-ray films in the dark and left at room temperature. The spots of acetylated and non-acetylated forms were cut and counted by liquid scintillation. Data were expressed as the percentage of acetylated chloramphenicol against non-acetylated chloramphenicol. The results of the mutant plasmids were expressed relative to the CAT activity of the wild type plasmid pT81.

2.7 Mobility Shift Assay

2.7.1 Preparation of C33A Nuclei

Nuclear extracts were prepared as described by Hennighausen and Luban (1987). Forty 100 mM plates of cultured C33A cells were washed and scraped in 1x PBS solution. The cells were harvested by centrifuging at 1800 rpm at 4°C in a Sorvall HB-4 rotor. The pellet of cells was resuspended in 10 volumes of chilled Dulbecco's phosphate buffered saline and centrifuged 5 volumes of 0.3 M sucrose in Buffer A was added to the pellet. Buffer A constitutes 10 mM HEPES-KOH at pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) and 2 µg/ml each of antipain, leupeptin and pepstatin A. The solution was transferred to a chilled Dounce glass homogenizer placed in ice. The cells were lysed with 8-12 strokes with a B pestle. Lysis was continued after 0.3-0.4% nonidet P-40 is added to the homogenate. The lysis of cells was monitored using a microscope. The homogenate was transferred to prechilled tubes and centrifuged at 2500 rpm at 4°C. The pelleted nuclei were washed twice in 0.3 M sucrose in buffer A without Nonidet P-40.

2.7.2 Preparation of Crude Nuclear Extracts

Nuclear extracts were prepared as described by Hennighausen and Luban (1987). The nuclei prepared by the method explained above were resuspended using an all-glass Dounce homogenizer in 2.5 pellet volumes of 400 mM NaCl, 10 mM HEPES-KOH at pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol and 0.5 mM PMSF. The nuclei were lysed with 10 strokes using a B pestle. The nuclei were gently stirred at 4°C for 30 minutes and centrifuged at 39,000 rpm for 60 minutes in a 75 Ti rotor. The supernatant was dialyzed against 50 volumes of 20 mM HEPES-KOH, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol and 0.5 mM PMSF, for 2-4 hours. The extract was then centrifuged at 20,000 rpm for 15 minutes to remove precipitate and lipid. The nuclear extract was aliquoted and frozen at -70°C after brief freezing in liquid nitrogen. The concentration of nuclear protein was estimated by the Bradford method (1976) using Bio Rad kit.

2.7.3 Preparation of Radiolabelled DNA Probes

The DNA fragments that were used to make probes for gel shift assays were generated by digesting the pUC19 plasmid containing the HPV 18 MCE with enzymes Hind III and Sac I. The deletion mutant enhancers and site directed mutant

enhancer were also obtained similarly. The fragments were electroeluted from 1% agarose gel. Radiolabelling of the probes was done by endlabelling the Hind III ends with $\alpha[^{32}\text{P}]\text{dCTP}$ by reverse transcriptase. The reaction mix was passed through Sephadex G-50 columns to remove unincorporated nucleotides from endlabelled DNA.

2.7.4 Protocol for Mobility Shift Assay

20,000 cpm of radiolabelled probe was used for each reaction. 300 fold excess of nonlabelled fragments were added to the reaction wherever required, as specific competitors. The EcoR I - Hind III fragment of pUC19 was used as competitor to the few flanking pUC19 bases present in the cloned enhancer fragments. 5 μg of nuclear protein and 5 μg of double stranded poly (dI.dC) were used in the reaction. The assay was performed as explained previously (Chodosh et al. 1988). The individual components were added in a final volume of 15 μl containing 4 μl of 12 mM HEPES-NaOH, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 10% glycerol, 0.6 mM DTT and 300 $\mu\text{g/ml}$ bovine serum albumin. The tubes were incubated at 30°C for 30 minutes. The labelled probe without nuclear extract was used as negative control. The reaction mixes were electrophoresed on 4% polyacrylamide gels at constant voltage, in a cold room. The circulation buffer contained 6.6 mM Tris-

HCl pH 7.5, 3.3 mM sodium acetate and 1 mM EDTA, which was constantly circulated using a pump. The gel was transferred to 3 mm filter paper, dried and exposed to x-ray film.

2.8 Protocol for UV Crosslinking Assays

The experiments were performed as described by Chodosh et al., (1988). Complementary strands of synthetic oligonucleotides with nucleotides 7667-7693 were annealed as described elsewhere (Kadonaga & Tjian, 1986).

The 27 mer wild type strands were: 5'-CTAAGTAATAAA ACTGCTTTTAGGCAC-3' and 5'-GTGCCTAAAAGCAGTTTATTACTTAG-3'.

The two mutant oligonucleotides used were: 5'-CTA AGTAATAAAAGTACTTTTAGGCAC-3' and 5'-GTGCCTAAAAGTACTTTTATTACT TAG-3'.

The annealed oligonucleotides were nick-translated with $\alpha(^{32}\text{P})\text{dCTP}$, 5-bromodeoxyuridine, DNase I, polymerase I and dNTP mix. After incubating for 60 minutes at 16°C, it was precipitated in 95% ethanol and 3M NaCl solution at -70°C by adding 5 μg salmon sperm DNA. The probes were washed by centrifugation, dried and used in UV crosslinking assays.

The reaction was done similar to mobility shift assay but with 75,000 cpm probe, 10 μg of protein and 10 μg of poly (dI.dC). At the end of the incubation, the reaction mixes were transferred to Nunc vials and covered with Saran

Wrap. The vials were placed under a Fotodyne UV lamp with emission wavelength of 310 nm and maximum intensity of 7000 $\mu\text{w}/\text{cm}^2$, for 30 minutes. To the vials, equal volume of 2x loading buffer containing 25 ml 4x Tris-HCl/SDS pH 6.8, 20 ml glycerol, 4 g SDS, 3.1 g DTT, 1 mg bromophenol blue in 100 ml was added. The vials were boiled for 3 minutes and loaded on to a 10% sodium dodecyl sulfate polyacrylamide gel. As a marker, commercially available prestained protein marker of high molecular weight range, comprising of myosin heavy chain - 200 kDa, phosphorylase b - 97.4 kDa, bovine serum albumin - 68 kDa, ovalbumin - 43 kDa, carbonic anhydrase - 29 kDa, β -lacto globulin - 18.4 kDa and lysozyme - 14.3 kDa was loaded.

CHAPTER 3

Results

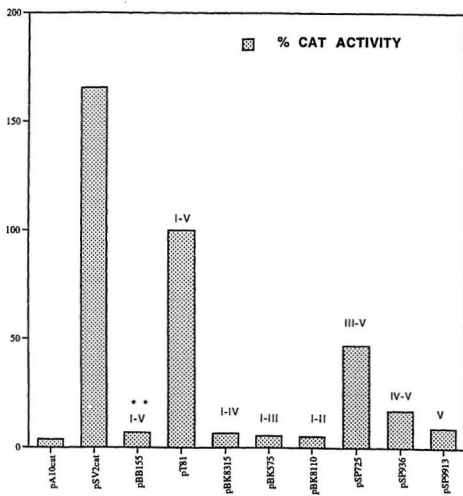
3.1 In Vivo Activities of the HPV 18 MCE Deletion Mutants in C33A Cells

In order to estimate the relative *in vivo* activities of the deleted regions of HPV 18 MCE with the full length MCE, the deleted regions were cloned upstream to the reporter CAT gene, transfected to C33A cells and CAT assays were performed. Since C33A cells do not contain HPV DNA, the transfected HPV 18 MCE regions are under the direct control of the host cellular factors.

The recombinant plasmid, pT81 was transfected by the calcium phosphate method into C33A cells. The plasmids pA10cat and pSV2cat were also transfected into the same batch of C33A cells each time, as negative and positive controls, respectively. CAT assays were performed to estimate the *in vivo* activities of the plasmids in C33A cells. The CAT reporter gene is driven by the full length HPV 18 MCE in plasmid pT81 and by SV40 enhancer in pSV2cat. In plasmid pA10cat, the enhancer is absent, with only the promoter present. CAT assay, therefore, was used to measure the enhancer activity in C33A cells.

Fig 5.

In vivo activities of deleted and mutant MCE relative to the activity of the full length 230bp HPV 18 MCE in C33A cells. The results were obtained from three experiments using DNA from two plasmid preparations. The results are presented as a % of pT81 activity. The corresponding regions of the MCE are listed above each bar. The asterisks indicate mutation generated by site-directed mutagenesis.



Plasmid pT81 showed high activity in the C33A cervical carcinoma cells. Since the plasmid does not contain an enhancer element, the activity of pA10cat was low(3.6 %). The activities of the deletion mutants in C33A cells were measured by transfecting the cells with the deletion mutant plasmids and performing CAT assays. The deletion mutants were cloned into pA10cat similar to the wild type plasmid pT81. This enabled the estimation of *in vivo* activities of the deleted regions of enhancer, relative to the function of the wild type enhancer as shown in Fig 5.

The 230 base pair MCE of HPV 18 (nucleotides 7508-7738) was designated into regions I to V as described previously (Nakshatri et al. 1990). The wild type plasmid pT81 had all the five regions intact. The CAT activity of pBK 8315 (nucleotides 7508-7664) was drastically reduced to only 6.5 percent relative to the activity of pT81. This plasmid had the MCE fragment with regions I to IV. The reduction in enhancer activity was most significant when region V was deleted. This indicated the importance of the region V in the MCE and factor(s) binding there in, to the overall activity of the HPV 18 MCE.

Plasmid pBK575 (nucleotides 7508-7640) showed 5.3 percent activity in comparison to pT81. This plasmid had regions I to III of the MCE. Similarly, plasmid pBK8110

(nucleotides 7508-7589, containing regions I-II expressed only 5.1 percent of the pT81 activity.

The activities of the plasmids containing MCE fragments deleted from region I towards region V were also measured by CAT assays. Plasmid pSP725 (nucleotides 7596-7738) contained regions III to V of the MCE. The activity was 46.9 percent of the full length MCE. The regions I, II and III were deleted and cloned in plasmid pSP936 (nucleotides 7632-7738). The CAT activity of this plasmid was 17 percent to that of pT81. Plasmid pSP9913 (nucleotides 7660-7738) containing only region V showed 9.16 percent activity as compared to pT81.

The activity of pSP9913 containing region V alone was almost twice that of plasmids containing the other regions but lacking the region V (pBK8315, pBK575, pBK8110), suggestive of the importance of region V in the HPV 18 MCE activity.

Since the plasmid pBK8315(nt 7508-7664) containing regions I-IV without region V showed a drastic reduction in activity, this indicates the importance of this region in the function of the HPV 18 MCE.

3.2 Gel Retardation Assays to Study DNA-Protein Interactions in Region V of HPV 18 MCE

After analysing the *in vivo* activity of the regions of MCE, the next objective was to investigate the DNA-protein

interactions involving the region V which has an important role in the activity of the MCE as demonstrated by the CAT assay results.

To investigate the role of DNA-protein interactions of region V with the cellular factors present in C33A cells, gel retardation assays were done using C33A nuclear extracts (see Fig 6). The full length 230 base pair MCE was also used to study the protein interactions.

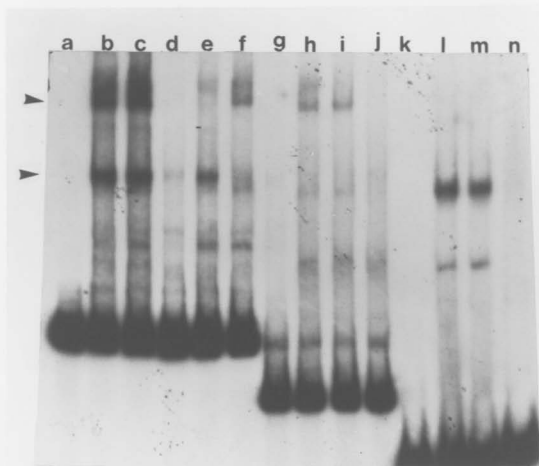
The deletion mutant enhancer fragment BK8315, which had nucleotides 7508-7664, was used to examine the effect of region V deletion on DNA-protein binding. This fragment had regions I-IV only. The fragment SP9913 containing nucleotides 7660-7738 has only the region V intact. This fragment was also used in gel retardation assays. The EcoR I - Hind III fragment of pUC19 vector was used as a competitor to the few bases of the polylinker region present flanking to the enhancer. In competition studies, nonlabelled fragments of BK8315 (regions I-IV) and SP9913 (region V) were separately added in reactions containing the labelled full length MCE probe to identify the specific binding to the region V.

Gel retardation of the HPV 18 full length MCE with C33A nuclear extracts consistently showed two distinct bands as shown in lane b in Fig 6. When nonlabelled MCE fragment was used as competitor, the bands were clearly competed (lane d). Competition with pUC19 polylinker sequence did not alter

Fig 6.

Gel retardation assay of HPV 18 MCE and deletion mutant enhancers BK8315 (regions I-IV) and SP9913 (region V) with C33A nuclear extract. 300 fold excess of non labelled competitor was added wherever indicated.

- a: 230bp HPV 18 MCE (wild type) probe alone.
- b: wild type probe with C33A nuclear extract.
- c: polylinker region of pUC 19 with b.
- d: nonlabelled wild type fragment with b.
- e: nonlabelled BK8315 fragment with b.
- f: nonlabelled SP9913 fragment with b.
- g: BK8315 probe alone .
- h: BK8315 probe with C33A nuclear extract.
- i: polylinker region of pUC 19 with h.
- j: nonlabelled BK8315 fragment with h.
- k: SP9913 probe alone.
- l: SP9913 probe with C33A nuclear extract.
- m: polylinker region of pUC 19 with l.
- n: nonlabelled SP9913 fragment with l.



the binding pattern (lane c), thereby demonstrating the specific binding of the proteins with the MCE sequences.

Binding assay with the BK8315 (regions I-IV) fragment showed only the slower migrating complex (lane h). This complex was competed when the nonlabelled BK8315 fragment was added to the reaction (lane j). The band was visible when competed with pUC19 polylinker fragment (lane i). When this BK8315 fragment was used as a competitor with labelled full length probe (lane e), only the faster migrating complex was present, whereas the slow migrating complex was absent. This (lanes h ,e) demonstrates the association of the slower migrating complex (upper) with regions I-IV of MCE.

When the SP9913 fragment containing only the region V was used, the slower migrating band was not observed (lane l). Only the faster migrating band was seen which was not affected by competition with pUC19 polylinker sequences (lane m), while competition with nonlabelled SP9913 did not show any band (lane n). When this region V fragment was used as competitor with the full length MCE (lane f), the faster migrating complex was greatly reduced, whereas the upper band was slightly reduced. This (lane l,f) demonstrates the binding of the faster migrating complex (lower) to region V.

The gel retardation assays demonstrated two distinct complexes binding to the 230 base pair MCE. The binding of region V was specific to the faster migrating band and the

slow migrating complex showed interactions with regions I to IV. The binding assays underlined the specific interactions of a protein complex with region V of HPV 18 MCE, correlating its *in vivo* activity in C33A cells. This demonstrates the binding of a protein complex to the region V , suggestive of its role in regulating the *in vivo* function of the region V.

3.3 *In Vivo* Activity of the Site Directed Mutant

With the identification of region V as an important region for the activity of the HPV 18 MCE , as demonstrated by *in vivo* CAT assays and the subsequent experiments on DNA - protein binding which indicates the specific association of C33A nuclear factors with region V , the first objective of the project was addressed. The next aim was to identify the sequence of this region V that is specifically involved in regulating the MCE activity.

The probable functional role of the ACTGCT sequence was speculated by the Pater's group (Nakshatri et al. , 1990). The presence of this sequence in the MCE of the oncogenic HPV 18 and HPV 16 and its absence in the non oncogenic HPV 11 was an interesting observation . This sequence is not known to be recognized by any transcription factor (Faisst & Meyer , 1992). Since the region V was found to be important for the activity of MCE , the ACTGCT sequence present at nt 7679 -

7684 was hypothesized to be essential. To elucidate the role of this sequence, site directed mutagenesis of this sequence was performed on the full length MCE. While designing the mutation, care was taken not to create a known recognition site. For an easier and efficient screening of the clones, a restriction site AGTACT for the enzyme Sca I was created.

This mutant plasmid pBB155, containing the mutant sequence AGTACT at nt 7679-7684 instead of the wild-type sequence ACTGCT, without changes in the rest of the full length MCE was transfected into C33A cells and CAT assays were performed (fig.5) to investigate the importance of this ACTGCT sequence in the HPV 18 MCE activity in C33A cells. The CAT activity of this mutant plasmid was drastically reduced to the extent similar to that expressed by deletion mutant plasmid pBK8315 (region V) as shown in Fig. 5. This demonstrates the importance of the ACTGCT sequence (nucleotides 7679-7684) in the *in vivo* activity of the HPV 18 MCE.

3.4 Gel Retardation Using the Site Directed Mutant

The demonstration of the important role in the regulation of the MCE activity by the ACTGCT (nucleotides 7679-7684), warranted further investigation in this regard. To address the involvement of DNA-protein interactions within

this short region of DNA, gel retardation assays were performed (Fig 7).

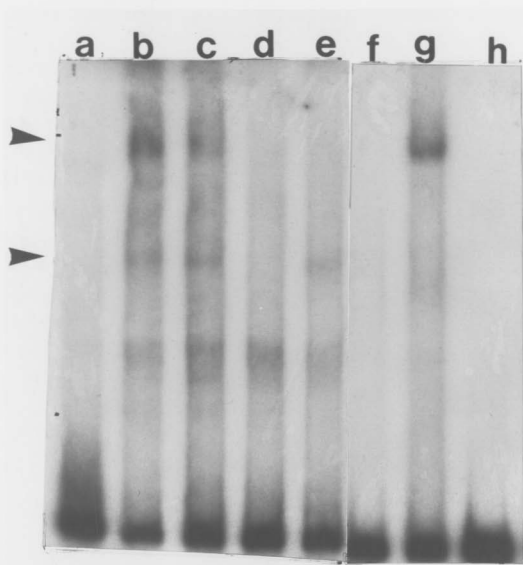
The 230 base pair full length MCE fragment was used in gel retardation experiment with C33A nuclear extract (lane b). Again, two distinct bands were present. When polylinker sequence of pUC19 vector was used as competitor, the bands were present (lane c). The bands were absent when non labelled full length MCE was used as specific competitor (lane d).

The addition of the mutant fragment BB155, which had the 230 base pair full-length MCE with mutations only at nucleotides 7680 and 7682, to the reaction (lane e) competed the slower migrating (upper) band but not the faster migrating (lower) band, indicating the inability of the mutant region to form a DNA-protein complex while the rest of the MCE binds to the proteins, thereby abrogating the slower migrating band only. When the mutant BB155 probe was used in gel retardation assay, only the slower migrating (upper) band was present (lane g). The binding pattern was similar to the gel retardation experiments done with the deletion mutant BK8315 containing regions I-IV (lane h, Fig. 6). The addition of non labelled mutant BB155 to the reaction competed the complex (lane h). This (lane e,g) demonstrates the specific binding of a cellular factor to the ACTGCT motif, which is abrogated by the site directed mutation.

Fig 7.

Gel retardation assay using wild type HPV 18 MCE and mutant BB155. 300 fold excess of non labelled competitor was added wherever indicated.

- a: wild type probe alone.
- b: wild type probe with C33A nuclear extract.
- c: polylinker sequence of pUC19 with b.
- d: nonlabelled wild type fragment with b.
- e: nonlabelled mutant BB155 fragment with b.
- f: mutant BB155 probe alone.
- g: mutant BB155 probe with C33A nuclear extract.
- h: nonlabelled mutant BB155 fragment with g.



3.5 UV Crosslinking to Identify the Factor Interacting with the Mutant Region

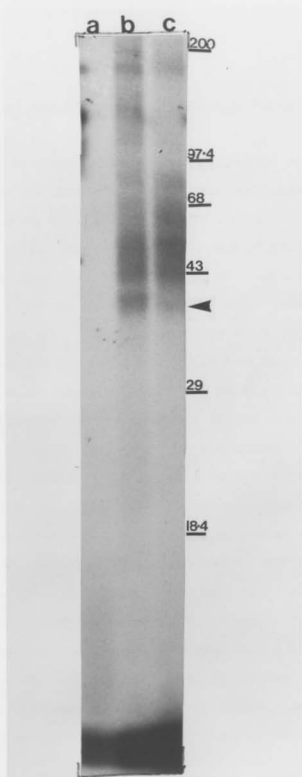
Since the gel retardation assays were done using the full length MCE and mutant MCE, UV crosslinking experiment was performed using synthetic oligonucleotides of nt 7667-7693, to further investigate the effect of the mutation in the DNA protein interactions (Fig 8). Complementary strands of wild type and mutant synthetic oligonucleotides containing nucleotides 7667 to 7693 were annealed, labelled and crosslinked with C33A nuclear extract.

While the wild type oligonucleotides demonstrated an intense band corresponding to a 40 kDa protein (lane b), there was an abrogation of this band when mutant oligonucleotide strands were used (lane c). While wild type oligonucleotides shows faint bands between 97 and 200 kDa, these were not consistently observed in repeated experiments. The absence of the 40 kDa band in the reaction with mutant oligonucleotides was a consistently reproducible observation, indicative of the inability of a 40 kDa protein to bind to the mutant sequence. Experiments with the addition of nonlabelled mutant oligonucleotide with labelled wild-type oligos would be a good control. An oligonucleotide with multiple repeats of the ACTGCT sequence can be used to identify the specific protein binding to this sequence.

Fig 8.

UV crosslinking of wild type and site directed mutant oligonucleotides with C33A nuclear extract.

- a: wild type probe alone.
- b: wild type probe with C33A nuclear extract.
- c: mutant probe with C33A nuclear extract.



CHAPTER 4

Discussion

4.1 Importance of the Minimal Constitutive Enhancer

The important role of the promoter and enhancer elements in the control of HPV 18 gene expression is well documented (Gius et al., 1988; Thierry et al., 1987; Butz & Hoppe-Seyler, 1993). The 230 base pair MCE of HPV 18 is known to be cell-type specific. The control of this enhancer does not involve any HPV 18 encoded gene product (Swift et al., 1987; Marshall et al., 1989). Since the C33A cells used in this project does not have HPV DNA integrated into the genome, the studies were performed without the overlaying effects of viral encoded factors on the function of the MCE.

HPV 18 is more efficient in the immortalization of epithelial cells than HPV 16. It is also associated with the rapid progression of invasive cervical carcinomas (Kurman et al., 1988). The increased immortalization potential of HPV 18 is believed to be conferred by its upstream regulatory region, which contains the MCE (Romanczuk et al., 1991). The interaction of cellular proteins with the enhancer element is well established (Garcia-Carranca et al., 1988; Gloss et al., 1989; Nakshatri et al., 1990). The importance of cellular factors in the regulation of HPV 18 expression attracted attention in the pursuit of understanding this molecular

mechanism. To address this question, the objective of this project was to identify the important region of the MCE and thereby the factor that plays a dominant role in this mechanism.

4.2 Significance of the Deletion Mutants

Unidirectional deletions of the MCE from either ends were generated to delineate the region most important for its function. Among all the deletion mutants, the deletion of region V showed the most reduction in activity. The plasmid pBK 8315 (nucleotides 7508-7664) containing region V had only 6.5 percent of the full length activity (Fig 5). This drastic reduction in the activity indicates the important role of region V of the MCE. Increased deletion of the enhancer towards region I did not show a further reduction in activity. An NF1 binding sequence is deleted in pBK8315. The reduced activity could be argued to be due to the absence of this NF1 binding to its motif. However, this is unlikely to be the reason, since NF1 is known to contribute only marginally to transcriptional activation (Gloss et al., 1989; Butz & Hoppe-Seyler, 1993). A Oct-1 binding site is also deleted in this mutant. But the inability of Oct-1 to bind would not lead to this reduced activity, since Oct-1 reportedly represses the enhancer activity (Mack & Laimins, 1991).

The deletion mutant pSP725 (nucleotides 7596-7738) containing regions III-V demonstrated 46 percent of pT81 activity. The deletion of two NF-1 binding sites present in regions I-II could not have contributed to this reduction, due to its known marginal contribution to the enhancer activity (Butz & Hoppe-Seyler, 1993). Interestingly, there is an ACTGCT motif at nucleotides 7545-7550, which has been deleted in mutant pSP725. This could be the possible reason for the slight reduction in the intensity of the upper complex in lane f of Fig 6, when region V fragment is used as a competitor with labelled full length probe. Since the importance of the ACTGCT sequence present at nt 7545-7550 has not been investigated by site-directed mutagenesis, it is not possible to analyse its involvement. However, the reduction in CAT activity is quite significant, since more than 50% of the activity is lost. In addition to the ACTGCT sequence, only two NF-1 binding sites are deleted in this mutant (pSP725). Both the NF-1 sites are half site NF-1 recognition motifs and are believed to be poorly bound by NF-1. Mutation of both the NF-1 sites did not lead to a strong reduction in the activity of the MCE, indicating the lesser role of these NF-1 sites (Butz & Hoppe-Seyler, 1993). This suggests a possible role of this ACTGCT sequence, which can be addressed by further studies.

The mutant pSP936 has regions IV-V (nucleotides 7632-7738). It expresses only 17 percent of activity relative to

pT81. In addition to the two NF-1 sites, ACTGCT motif, an AP1 site is also deleted (Offord & Beard, 1990). AP1 sites are known to be essential for the HPV 18 MCE activity (Thierry et al., 1992). A recent study demonstrated the role of Jun B as the predominant Jun component complexing with AP1 elements. Mutational analysis of this AP1 site established its importance in HeLa and HaCAT cells. However, mutation of the AP1 site resulted in a less drastic reduction in the transcriptional activity in C33A cells than in keratinocytes. It was suggested that in addition to the AP1, a yet to be identified factor is important in C33A cells (Offord & Beard, 1990; Thierry et al., 1992; Butz & Hoppe-Seyler, 1993). The mutant pSP9913 containing region V (nucleotides 7660-7738) has binding sites for NF1 and Oct-1. The reduced activity could be due to the deletion of two Oct-1 sites, two NF1 sites and AP1 element (Mack & Laimins, 1991; Gloss et al., 1989; Offord & Beard, 1990). Also, an octomer motif at nucleotides 7731-7738 in region V has been reported to inhibit HPV 18 expression in cervical cells by inhibiting the binding of NF1 to its site at nucleotides 7731-7735 (Morris et al., 1993).

It is important to note that the activity of the plasmid pSP9913 containing the region V alone is almost double that of the plasmids (pBK8315, pBK575, pBK8110) containing other regions of the MCE but without region V.

The *in vivo* assays using deletion mutants clearly demonstrate the importance of region V and is suggestive of the role of a novel factor present in C33A cells, in the regulation of HPV 18 MCE expression.

The gel retardation assays using the wild type HPV 18 MCE and deletion mutants were performed to investigate the DNA- protein interactions involved in the region V of the MCE (see Fig 6). Since the deletion mutant BK8315 has regions I-IV, the use of this fragment would show the interaction of proteins with these regions. The use of SP9913 fragment containing region V only was designed to demonstrate the association of proteins with this region. Accordingly, two complexes were seen with the wild-type full length MCE (lane b). When the BK8315 fragment was used only the slower migrating complex was observed (lane h). The reaction using SP9913 demonstrated only the faster migrating complex (lane l). Competition experiments were performed to identify the specificity of the complex formation with the regions of MCE. When C33A nuclear extracts were incubated with nonlabelled BK8315 fragment before adding the labelled wild-type probe, only the faster migrating complex was seen (lane l). This demonstrates the binding specificity of the slower migrating complex to regions I-IV of the MCE. Similarly, when nonlabelled SP9913 fragment was included in the reaction with labelled wild-type probe, the slower migrating complex was

abrogated while the faster migrating complex was observed (lane f). This shows the specific binding of region V to the faster migrating complex. To rule out the possibility of non specific binding, homologous nonlabelled fragments were added in the reactions (lane d,j,n). Nonlabelled pUC19 polylinker sites were added as competitors to demonstrate the specific binding of factors to the enhancer sequences (lane c,i,m).

The use of deleted fragments with regions I-IV (BK8315) and region V (SP9913) along with wild-type full length HPV 18 MCE in gel retardation assays using C33A nuclear extracts demonstrated the formation of a specific DNA -protein complex with region V.

4.3 Site Directed Mutagenesis Confirms the Importance of a Novel Factor

After delineating the region V as the important region in the functional regulation of HPV 18 MCE activity, the next objective was to identify the sequence involved in this regulation. Work done by the Pater's lab had speculated on the possible role of the ACTGCT sequence in the function of the HPV 18 MCE (Nakshatri et al.,1990). Since this sequence is also present in HPV 16, but absent in the non oncogenic HPV 11, the pursuit of investigation on this sequence seemed attractive. The suggestion by Thierry et al., (1992), on the

involvement of a novel factor in the regulation of HPV 18 MCE in C33A cells is supported by the findings in the present work, since no known transcription factor binds this sequence (Faisst & Meyer, 1992). However, the findings of the experiments performed in this study does not limit the boundary of this motif to only six nucleotides.

The *in vivo* activity of the mutant plasmid pBB155 was similar to the activity of the deletion mutant pBK8315. Since only the ACTGCT motif was mutated without altering the other sequences of the HPV 18 MCE, this motif appears to have an important role in the regulation. UV crosslinking (Fig 8) with C33A nuclear extract demonstrates the inability of a factor to bind to the mutant motif, thereby indicating the importance of the ACTGCT motif for the interaction of a protein factor with the DNA sequence.

Since the mutations do not create a change in the spacing between nucleotides, a control construct with two point mutations elsewhere in the MCE was not generated. Though a reasonable rationale exists to perform this control the generation of such mutant was not pursued. To facilitate a better screening efficiency, the design of the mutation was to create the recognition site AGTACT for the restriction enzyme ScaI. Interestingly, the sequence created is a palindromic sequence. The presence of a palindrome could lead to binding of factors in dimers. Since the gel retardation assay and uv

cross linking experiment demonstrated the abrogation of a complex, this can be ruled out. The presence of a perfect palindromic sequence in a double stranded DNA could lead to the formation of a pair of hairpinned structures called cruciform (Platt, 1955). It is plausible to believe that this formation can prevent the binding of a protein by structural inhibition rather than the change in the sequence. However, elegant experiments by Courey & Wang, (1983) have demonstrated that cruciform does not occur under physiological conditions. Isolated plasmids containing 68 bp perfect palindrome were found to be free of cruciform. The plasmid lacked cruciform even *in vivo*. Therefore it is unlikely that this is happening with the mutant plasmid used in this project. Moreover, the mutant sequence AGTACT creates a ScaI recognition site which was utilized in the screening procedure. Only plasmids digested by ScaI enzyme were used for further cloning and transfection. Therefore the reduction in the *in vivo* activity and binding could be only due to the change in the wild type ACTGCT sequence and not due to cruciform formation.

A recent study on the role of AP1 elements on HPV 18 transcription has outlined its importance (Thierry et al., 1992). However, it was observed by the authors that in C33A cells the transcription was efficient, despite the low amounts of AP1 in the cells. It was speculated that an unidentified factor played a predominant role in C33A cells. The

suggestion was that Jun B would interact with an "unidentified transcriptional factor " in regulating HPV 18 expression. The cell type specificity of HPV 18 expression is believed to be regulated by a multifactor phenomenon (Thierry et al., 1992). A similar hypothesis has been proposed by Ishiji et al. (1992) in the cell type specific expression of HPV 16. The protein identified as transcriptional enhancer factor (TEF)-1 was shown to complex with the HPV 16 enhancer. A "TEF-1 coactivator" was believed to be associated with TEF-1 in the regulation of HPV 16 expression in keratinocytes and cervical carcinoma cells (Ishiji et al., 1992). The data presented here suggest an important role of the ACTGCT motif and indicates the involvement of an "ACTGCT binding protein" (ABP) in the regulation of HPV 18 expression. This appears to be a novel factor with a significant role in the regulation of HPV 18 transcription in C33A cervical carcinoma cells. This factor could mediate the regulation of HPV 18 expression, by its specific binding to the ACTGCT motif and also by its involvement in the assembly of the protein complex, that might be required for the full expression. Further experiments will elucidate the exact mechanism involved in the regulation of HPV 18 expression.

The cellular factors NF- κ B, Oct-1, AP-1 are known to be ubiquitous in their distribution, while the expression of HPV 18 is evidently tissue specific. It is therefore logical to

infer that this tissue tropism is not regulated by these cellular factors. Though KRF-1 is hypothesized to be involved in the tissue tropism, the differential expression of HPV 18 with mutated KRF-1 site in HeLa and HaCat cells was not correlated with a difference in the binding pattern of KRF-1. The mutation of KRF-1 site did not lead to a strong reduction in the transcriptional activity of HPV 18 in C33A cells (Butz & Hoppe-Seyler, 1993). It has been speculated by Mack & Laimins, (1991) on the possible role of other unidentified factors in stabilising and activating transcription of HPV 18. Given these findings, the importance of the "ACTGCT binding protein" appears to be attractive.

Cotransfection of Jun B expression vector with reporter gene under the control of HPV 18 URR failed to show expression in fibroblasts (Thierry et al., 1992). This indicates the inability of Jun B to activate HPV 18 expression in non epithelial cells. The expression of HPV 18 was found to be very low when pT81 was transfected into 143B fibroblast cells. This could be due to the lack of the ACTGCT binding protein in 143B cells.

The products of early genes E6 and E7 of HPVs are known to be the oncoproteins involved in the transformation and tumor formation. The E6 and E7 gene products of high risk HPVs are adequate for immortalizing primary rodent cells, while these genes of the low risk HPVs fail to immortalize. E7

alone is capable of immortalization unlike the E6 gene product which requires E7 for the successful immortalization. In cooperation with activated Ras oncogene, the HPV oncoproteins can induce a fully transformed phenotype resulting in tumor formation in mice. In association with Ras, E7 alone can cause transformation and is required for maintenance of the transformed phenotype. The infection of target cells with HPV and the subsequent expression of the oncoproteins is believed to be the first step towards tumor formation. The underlying mechanism involved in this process is believed to be due to the binding of E6 and E7 to p53 and pRb. The E6 protein from low risk HPVs does not lead to degradation of p53 like the E6 protein from HPV16 and HPV 18. Unlike the E7 proteins from the high risk HPVs, the E7 products of low risk HPVs bind pRb with lesser affinity. The expression of E6 and E7 genes is regulated by the E2 gene product. In benign lesions the HPV genome is found in episomal form, while the HPV genome integrates into the host genome in most malignant lesions (Mansur & Androphy, 1993; Munger et al., 1991; Gius et al., 1988). The integration of the HPV genome leads to the disruption of the E2 gene. The E6 and E7 are then regulated by the upstream regulatory region (URR) which has binding sites for many cellular factors (Garcia-Carranca et al., 1988).

Since the balance of the E2 products leads to inhibition of transcription, the disruption of this control is

believed to lead to malignant change in the cells (Ward et al., 1989). This increase in expression of oncoproteins is mediated by the cellular factors that bind to the URR. It is therefore important to understand the role of cellular factors that interact with the MCE. It could be speculated that ABP has an important role in this multifactor mechanism.

The cellular transcription factor Sp1 is believed to be involved in the biology of papillomaviruses. The binding of E2 protein to the HPV 18 URR is known to sterically interfere with stable formation of the preinitiation complex. Sp1 is known to displace the binding of E2 and thereby leading to the activation (Hoppe-Seyler & Butz, 1994). It could be speculated that ABP contributes to the stabilization of the transcription machinery by interacting with the adjacent cellular factors.

The HPV 18 URR has a binding site for a cellular zinc-finger protein YY1 close to the GRE. The binding of the YY1 factor to this negative regulatory region of the HPV 18 URR leads to repression of the E6/E7 promoter. The YY1 binds to the silencer element with high affinity to negatively regulate the E6/E7 promoter. This repression appears to be overcome in some carcinomas by mutation or deletion of this region (Butz & Hoppe-Seyler, 1993; Bauknecht et al., 1992). It is tempting to speculate the possible role of ABP in inactivating this silencer whenever it is present without alteration, thereby leading to the expression of E6 and E7.

This could be one of the mechanisms employed by the virus to escape cellular control and thereby cause malignant transformation.

In summary, the ACTGCT motif (nucleotides 7679-7684) present in the HPV 18 MCE appears to be important and indicates the role of a novel ACTGCT binding protein (ABP) in the regulation of HPV 18 expression in C33A cells. Though the presence of the ACTGCT sequence alone is not sufficient to activate transcription, the mutation of this binding motif leads to a drastic reduction in the activity, suggestive of its requirement for the overall activity of the HPV 18 MCE.

Future Directions

The significance of this protein needs to be further investigated. While the present work deals with the ACTGCT sequence present at nt 7679-7684, it would be interesting to mutate the ACTGCT sequence of HPV 18 MCE present at nt 7545-7550 and study its activity. Both *in vivo* and *in vitro* changes need to be estimated. A plasmid with mutations in both ACTGCT sites and AP1 element would be useful in knowing the interactions between the two factors. Since the mutation of AP1 alone does not lead to a significant change in the HPV expression in C33A cells, the requirement of the novel factor can be demonstrated.

The isolation and characterization of this factor from C33A cells will be useful. An oligonucleotide with multiple repeats of ACTGCT motif can be used as the probe to screen a C33A cDNA expression library. Functional analysis of the protein will further aid in the understanding of its role in transcriptional regulation.

An ACTGCT motif is present in the HPV 16 MCE also. No factor is known to bind at this site (Nakshatri et al., 1990). Gel shift assay using that region will indicate the presence of a DNA-protein complex. Further analysis by site directed mutation can also be done. These studies would help in understanding the role of this motif (and the factor) in the regulation of HPV 16 MCE in addition to its role in HPV 18 MCE which is evident from the present study.

Immortalized cells of early passage can be used for transient assays to look into the *in vivo* activity of HPV 18 MCE in these cells. Comparative studies could be done on the role of ABP by performing CAT assays using both the wild-type plasmid and mutant plasmid using cervical carcinoma cell lines and also in immortalized cells of early passage. Simultaneous binding assays should be done. The status of HPV DNA in the host cells must be analyzed. The effect of the transformation status of cells on the regulation of HPV 18 MCE by ABP can be addressed by these experiments.

The effect of the factor on HPV 18 expression could be due to the differential distribution of the protein. Mobility shift assays using nuclear extract from different cell types will indicate whether the protein is ubiquitous or cell specific. The (increased)/presence of this factor might be a cause or effect of malignant transformation/HPV expression. The cDNA of the gene can be used in cotransfection assays in cells that do not activate HPV 18 expression/lack the protein.

The understanding about the regulatory role and tissue distribution of this protein might help in elucidating the mechanism of HPV 18 expression and malignant transformation. With the knowledge about this process, the clinical relevance of this factor can be understood.

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